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Nutrient Translocation during Early Disc Regeneration in the Brittlestar *Microphiopholis gracillima* (Stimpson) (Echinodermata: Ophiuroidea)

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Abstract. *Microphiopholis gracillima* can autotomize and then regenerate the autotomized central disc, including integument, gut, and gonads. Experiments were carried out to determine the relative importance of internal nutrient reserve translocation and exogenous nutrient uptake during the regeneration process. Approximately 60% of the dry body weight of *M. gracillima* is organic material. Intact animals held for three weeks in natural seawater did not change significantly in weight, caloric content, or relative concentration of protein, carbohydrates, or lipids. Intact animals held for three weeks in artificial seawater devoid of nutrients lost weight and caloric content. The rate of loss was rapid initially, but slowed after about eight days. Animals regenerated in natural seawater lost weight initially, then regained the lost weight. Animals regenerated in artificial seawater lost weight constantly and at a higher rate than either the artificial seawater control or natural seawater regenerated animals. All weight losses were attributable to significant changes in the protein and carbohydrate fractions of the organic body component. The lipid fraction and ash components did not change significantly in any treatment. *M. gracillima* appears to be adapted to regenerate the lost disk rapidly, even under conditions of food deprivation.

Introduction

Autotomy (self-mutilation by casting off body parts), followed by regeneration of the lost parts, is widespread among the echinoderms, and these animals are known to have superb wound-healing and regenerative capacities (see review by Emson and Wilkie, 1980; Brown, 1982). The few published studies of echinoderm regeneration have dealt almost exclusively with the capacity of an individual species to regenerate, descriptions of the appearance of new structures, or measurements of regeneration rates (Gibson and Burke, 1983). Although two recent studies have estimated the environmental energy production represented by regenerating brittlestar arms (Duineveld and Van Noort, 1986; O'Conner *et al.*, 1986), the energetic costs to the regenerating animal of autotomy, and the sources of nutrition for regeneration in echinoderms, have not been evaluated.

Members of at least five families of ophiuroid echinoderms (brittlestars) autotomize arms or the aboral disc (including digestive tract, gonads, and disc epithelium) when disturbed. They can regenerate these tissues within a few weeks in the laboratory (Emson and Wilkie, 1980; pers. obs.). The rate of tissue replacement must be related to the amount of stored nutrients available and the rate at which the animal can accumulate and allocate additional nutrients for tissue regeneration. The ophiuroid disc begins to regenerate before the gut has been replaced, but the sources of the nutrients that support that process are uncertain. To date, no specific nutrient storage organ other than the disc has been found, although the interstices of the arm ossicles may be repositories for nutrients (Turner and Murdoch, 1976). The ability of many echinoderms to translocate nutrients during gametogenesis (Lawrence, 1987) suggests that the same mechanism could be used during regeneration. Echinoderms may also take up dis-

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solved organic matter (DOM) from the surrounding seawater, or process exogenous particulate nutrients by external digestion (Lawrence, 1987; Clements, 1988).

Theoretically, use of either DOM alone or external digestion alone would result in a net gain of organic matter by regenerating animals with no concomitant tissue loss from non-regenerating portions of the body. In contrast, internal nutrient translocation would result in the decrease of tissue in non-regenerating portions of the animal, with no net gain of organic matter during regeneration. There should even be a net loss of tissue due to catabolism of tissue constituents for respiration during regeneration. But translocation of stored nutrients, external digestion, and DOM uptake are not mutually exclusive and may operate sequentially or simultaneously. Other echinoderms lacking special storage organs resorb body parts under starvation conditions (Ebert, 1967; Feral, 1985; Lawrence, 1987). We hypothesize that early disc regeneration (prior to reformation of the functional gut) relies heavily on translocation of internal nutrient stores that are mobilized from the non-regenerating somatic tissues. The purpose of this paper is to estimate the relative contribution of internal nutrient translocation to disc regeneration.

Material and Methods

Individuals of *Microphiopholis gracillima* were collected from intertidal mud flats in the North Inlet Estuary just north of Georgetown, South Carolina (37°20′N, 79°10′W). After collection, animals were taken to the laboratory and sorted to eliminate all but individuals with complete (or almost completely regenerated) discs. Animals were then placed in autoclaved all-glass aquaria in an environmental chamber held at 25°C with a 12:12 light:dark cycle. The aquaria contained Millipore-filtered (0.45 μm) natural seawater (30‰). All aquaria were constantly aerated. The seawater was changed daily to control bacterial contamination (Clements *et al.*, 1988). Field-collected animals may have large differences in their nutritional states; therefore all animals were allowed to acclimate to the above conditions for seven days before the start of the experiments to help equalize nutritional differences. Animals were then randomly assigned to experimental groups.

The amount of nutrients translocated during regeneration was estimated in the following treatments. Intact and autotomized individuals were held in autoclaved all-glass aquaria containing either Millipore-filtered (0.45 μm) natural seawater without sediment; artificial seawater alone (Cavanaugh, 1956; trace minerals formula 5); or artificial seawater with approximately 125 μmol/l glucose, 125 μmol/l palmitic acid, and 12.5 μmol/l of each of 21 amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) added, for a total DOM concentration of approximately 513 μmol/l. This represents about a five-fold increase over natural DOM levels. (Clements, 1988; Williams, 1975). All aquaria were constantly aerated and kept in an environmental chamber at 25°C with a 12:12 light: dark lighting regimen. All media were changed daily. At 4-day intervals, 10 animals were removed from each treatment and dissected into the following body fractions: proximal, medial, and distal thirds of the arms, the oral frame, and the regenerated (or intact) discs. All of these fractions were dried to constant weight *in vacuo* over anhydrous calcium carbonate. About half of each fraction was ashed at 400°C for 6 h and its ash-free dry weight determined. The remaining three parts were subjected to biochemical analyses for protein, carbohydrate, and lipid, respectively. Before biochemical analysis, each part was split into three replicates, weighed, and ground to a dry powder in a hand-held all-glass homogenizer. In this way, replicated estimates of protein, carbohydrate, and lipid were obtained for each body fraction, as well as estimates of ash-free dry weight and caloric content. Because the tissue samples were small, colorimetric techniques were used in the biochemical analyses.

Total carbohydrate levels (as reducing sugars) were estimated by the phenol-sulfuric acid method of Dubois *et al.* (1956), with a 1:1 glucose:maltose solution as the standard. Total proteins were quantified by the Bio-Rad (Richmond, CA) modification of the Bradford (1976) method, with a 1:1 mixture of bovine serum albumin and purified mollusk protein (Sigma) as the standard. Lipids were extracted from the fraction homogenate with a 2:1 (v:v) chloroform:methanol solution. The extract was processed according to the sulphophosphovanillin method of Barnes and Blackstock (1973), with purified *Microphiopholis gracillima* lipid as the standard. The purified standard was prepared according to the procedures outlined in Barnes and Blackstock (1973). To compensate for the possibility of significant variation in total weights of the specimens used in this study, and to control for the inevitable loss of tissue during the fractioning of the samples for the different biochemical assays, all biochemical measures are reported as units per gram dry weight of specimen rather than as absolute quantities per body part.

To determine the relative translocation rate of proteins, carbohydrates, and lipids during regeneration, 300 animals were incubated in artificial seawater with either ^{14}C-leucine (specific activity 348.0 mCi/mmol), ^{14}C-glucose (3.5 mCi/mmol), or ^{14}C-palmitic acid (850.0 mCi/mmol) added at concentrations of 0.03 μCi/ml, 0.04 μCi/ml, and 0.04 μCi/ml, respectively. After 48 h, the animals were removed from the medium and rinsed in several changes of artificial seawater. One half of the animals in each nu-

trient treatment were induced to autotomize following the procedure of Dobson (1984, 1985). Five animals from each of the six treatments were immediately processed (see below). The remaining specimens were held in autoclaved all-glass aquaria containing constantly aerated artificial seawater in an environmental chamber at 25°C with a 12:12 light:dark cycle. The seawater was changed daily. At 4-day intervals, for 20 days, 8 animals were removed from each treatment and dissected to separate the distal half of the arms, the proximal half of the arms (including the oral frame), and the regenerated (or intact) disc tissue. Each fraction of five individuals was dried to constant weight at 80°C and placed in a separate glass scintillation vial containing 1 ml of 1:1 ProtoSol (New England Nuclear) tissue solublizer:ethanol. Five ml of AquaSol liquid scintillation cocktail (New England Nuclear) was added to each solublized specimen vial, and the samples were counted with a Beckman liquid scintillation counter with internal quench correction. The other three individuals were dried to constant weight at 80°C, ashed at 400°C for 6 h, and weighed again. We normalized all counts by computing the counts per minute (CPM) per gram dry weight and per gram ash-free dry weight of the tissue.

All data were analyzed by one-way or two-way ANOVA, Tukey's multiple comparison procedure (Ostle and Mensing, 1975; Sokal and Rohlf, 1981), or least-squares linear regression using the General Linear Models Procedure of the Statistical Analysis System (Carey, North Carolina). The probability of making any type I error at all in the entire series of tests was held at a = 0.05 or less [= Experimentwise error rate (Sokal and Rohlf, 1981, pg. 241)].

Results

Biochemical composition of the intact brittlestar

Normal values for organic and inorganic constituents of whole and individual regions of *Microphiopholis gracillima* were obtained by pooling all of the initial (time = 0) biochemical measurements from each experiment. The results are summarized in Table I. About 60% of the total dry body weight is organic tissue (as ash-free dry weight), and most of it is located in the arms. The central disc has the highest organic content (74%) relative to inorganic material, but this represents only 7% of the total dry body weight and 10% of the total organic tissue weight. The proximal, medial, and distal arm parts and the oral frame region contain 50 to 60% organic material, which accounts for 90% of the total organic material. The arms have a higher percentage of organic material at their bases than at their tips.

The central disc and oral frame have higher concentrations (per gram dry weight) of all organic components than do any of the arm regions. The disc has the highest concentration (per gram dry weight) of protein and lipid, whereas the oral frame has the highest concentration of carbohydrates. All arm fractions are similar in their protein, carbohydrate, and lipid concentrations. Interestingly, the assayed total protein, carbohydrate, and lipid content of the body accounts for only 30% of the total ash-free dry weight (= organic content) of the brittlestar. The relative underrepresentation of organic material is constant between body fractions with the exception of the oral frame, which has a relatively lower underrepresentation. Most of the total missing organic material is located in the arm parts. Although colorimetric assays commonly underestimate the actual amount of material present (Dubois *et al.*, 1956; Barnes and Blackstock, 1973; Davis, 1988), the magnitude of the underrepresentation in this case is unusual. We assume that, as has been reported for other echinoderms (Geise, 1966; Feral, 1985), the majority of the missing material represents insoluble organic material (such as connective tissue), organics tied up in the stromal spaces of the ossicles, complexed biochemicals (*e.g.*, glycoproteins and lipoproteins) that were not detected by the assays, and nucleic acids.

Change in biochemical composition of tissues during regeneration

Body weight changes. The changes in total dry weight (DW), total organic weight (= ash-free dry weight, AFDW) and total inorganic material weight (=ASH) fractions with time in individuals in the natural seawater control (NC), artificial seawater control (AC), natural seawater regenerated (NR), and artificial seawater regenerated (AR) treatments are shown in Figure 1. Animals in artificial seawater with added organics did not survive the experiment and thus were not analyzed. Animals in the NC group did not exhibit any significant change in total DW ($P = 0.3919$), ASH weight ($P = 0.9406$), or AFDW ($P = 0.4805$) during the course of the experiment (Fig. 1A). AC animals showed a rapid initial drop in both total DW ($P = 0.0466$) and AFDW ($P = 0.0002$) until day eight, after which both weight measures remained relatively constant. Total ASH weight did not change significantly at any time in the AC group ($P = 0.0828$) (Fig. 1B). NR animals did not lose significant amounts of total DW ($P = 0.0546$) or ASH weight ($P = 0.4458$) with time, but gradually lost AFDW ($P = 0.0022$) until about day 12, after which AFDW gradually increased through day 20 (Fig. 1C). The NR and AC groups lost as much as 40% of their initial AFDW values at some point during the 20-day experiment. AR animals displayed a rapid initial drop in total DW ($P < 0.0001$) and AFDW ($P < 0.0001$) from day 0 to day 4, followed by a slower constant decrease in these values. The maximum loss of DW during the ex-

Table I

Normal biochemical composition of Microphiopholis gracillima

Constituent	Whole	Disc	Body Part: Proximal arms	Medial arms	Distal arms	Oral frame
DRY WEIGHT	91.32 ± 10.87	8.85 ± 1.44	29.27 ± 3.57	27.50 ± 4.76	22.32 ± 4.71	2.26 ± 0.26
(% total body part DW)	100	100	100	100	100	100
(% whole DW)	100	9.7	32	30.0	24.5	2.4
ASH-FREE DRY WEIGHT	52.47 ± 7.78	6.52 ± 1.05	15.96 ± 2.64	16.32 ± 2.87	12.35 ± 2.58	1.35 ± 0.22
(% total body part DW)	100	74	54.5	59.3	55.3	59.7
(% whole DW)	58	7.1	17.5	17.8	13.5	1.47
(% whole AFDW)	100	12	30.4	31.1	23.5	2.5
ASH WEIGHT	37.70 ± 4.81	2.33 ± 0.42	13.31 ± 1.30	11.19 ± 3.72	9.97 ± 2.14	0.91 ± 0.14
(% total body part DW)	100	26	45.5	40.6	44.6	40.3
(% whole DW)	42	2.6	14.6	12.3	10.9	0.9
(% whole Ash weight)	100	6.2	35.4	29.7	26.4	2.4
PROTEIN	6.53 ± 0.35	1.59 ± 0.18	2.04 ± 0.05	1.44 ± 0.03	1.17 ± 0.09	0.21 ± 0.01
(% total body part DW)	7.15	18.02	7.00	5.27	5.27	9.60
(% whole DW)	7.15	1.74	2.23	1.57	1.28	0.23
(% total protein)	100	24.35	31.24	22.05	17.91	3.21
CARBOHYDRATES	2.88 ± 0.26	0.74 ± 0.07	0.91 ± 0.13	0.65 ± 0.14	0.33 ± 0.04	0.11 ± 0.01
(% total body part DW)	3.15	8.42	3.08	2.37	1.48	8.80
(% whole DW)	3.15	0.81	0.99	0.71	0.36	0.12
(% total carbohydrates)	100	25.69	31.59	22.56	11.45	3.81
LIPIDS	3.46 ± 0.33	0.74 ± 0.05	1.05 ± 0.24	0.94 ± 0.08	0.60 ± 0.17	0.10 ± 0.01
(% total body part DW)	3.79	8.36	3.58	3.41	2.68	4.50
(% whole DW)	3.79	0.81	1.14	1.02	0.65	0.11
(% total lipids)	100	21.40	30.60	27.10	17.60	2.89
UNACCOUNTED ORGANICS	39.60 ± 3.89	3.45 ± 0.09	11.96 ± 0.96	13.29 ± 1.13	10.25 ± 0.76	0.42 ± 0.02
(% total body part DW)	43.36	38.90	40.86	48.30	45.93	18.58
(% whole DW)	43.36	3.77	13.09	14.50	11.22	0.46
(% whole AFDW)	65.47	6.57	22.79	25.32	19.53	0.80
(% total UO)	100	8.71	30.20	33.56	25.88	1.06
CALORIC CONTENT (calc.) (kCal/g dry weight)	0.93 ± 0.04	2.40 ± 0.06	0.83 ± 0.08	0.70 ± 0.03	0.58 ± 0.08	1.08 ± 0.07

All values are averages ± one standard deviation. All units are milligrams unless otherwise noted.
DW = Dry Weight, AFDW = Ash-Free Dry Weight, UO = Unaccounted Organics.

periment occurred in this group, which lost as much as 40% of the initial DW and 50% of the initial AFDW. Ash weight did not change significantly in the AR group ($P = 0.4893$) (Fig. 1D).

When the total DW measurements were broken down by body part, the following trends were observed. In the NC group, no significant DW change occurred in any body part with time ($P > 0.05$ in all fractions) (Fig. 2A). In the AC group, the DW of the medial ($P = 0.4247$) and proximal ($P = 0.4928$) regions of the arms remained relatively constant, but the disc, distal arm regions, and oral frame lost DW until about day eight, after which their dry weights remained constant ($P = 0.0003$, $P = 0.0029$, $P = 0.0025$, respectively) (Fig. 2B). Animals in the NR group exhibited no overall change in DW in any body part ($P > 0.05$) after first appearance of the disc tissue, although the weight of the oral frame on day 16 was significantly different from all other days. Animals in the AR group lost DW throughout the experiment in all non-regenerating body parts ($P < 0.05$). This loss was rapid until approximately day eight, after which the decline proceeded at a slower rate.

Ash-free dry weight and ASH weight measurements by body part with time indicate that the loss in DW is due to loss exclusively from the organic fraction (Fig. 3). There were no significant changes in the ASH weights of any body parts in any experimental treatment over the 20-day period with the exception of first appearance of the discs (between days zero and four) in the regenerating groups (Fig. 4). There was no significant change in AFDW in anybody part with time ($P > 0.05$) in the NC group (Fig. 3A). In the AC group, all body parts with the exception of the proximal arm fractions lost AFDW until approximately day eight, after which AFDW remained relatively

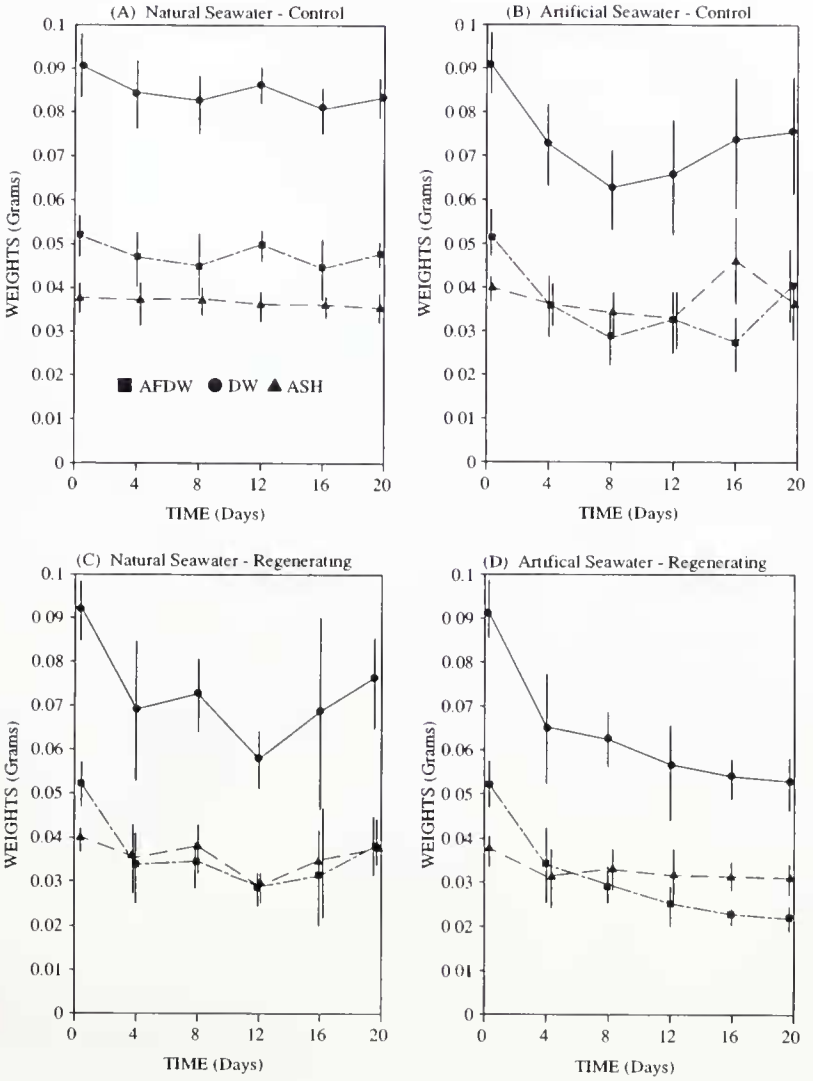

Figure 1. Total body weight changes during early disc regeneration. (A) Natural seawater control group. (B) Artificial seawater control group. (C) Natural seawater regenerating group. (D) Artificial seawater regenerating group. Error bars represent 95% confidence intervals. Error bars and points offset slightly for graphical clarity.

constant in all body fractions (Fig. 3B). The most rapid drop in AFDW occurred between day zero and day four. Although the proximal arm fractions did lose AFDW over the course of the experiment, the loss was not significant at any time ($P = 0.1443$). Animals in the NR group lost AFDW from all non-regenerating body fractions until approximately day 12, after which AFDW increased (Fig. 3C). Because of the high variability in the data, the changes in AFDW of the proximal and medial arm fractions were not statistically significant from day zero at any other time ($P = 0.0566$ and $P = 0.0853$, respectively). The AFDW of all non-regenerating body part fractions in the AR group declined continuously until day 16 of the experiment ($P < 0.05$) (Fig. 3D). The most rapid decrease occurred between day zero and day four, except in the proximal arm regions, where tissue was lost at a constant rate. The disc tissue in both the NR and AR groups did not increase in AFDW content significantly after first appearing.

Protein content changes. The changes in total body protein concentration over time are shown in Figure 5A. The natural seawater control group did not change in total protein concentration over the course of the experiment ($P = 0.4717$). The artificial seawater control group exhibited a slight decline in protein concentration with time ($P = 0.0121$), but the only day that was significantly different from the others in this group was day eight. The groups regenerating in natural seawater and in artificial seawater both changed slightly in total protein concen-

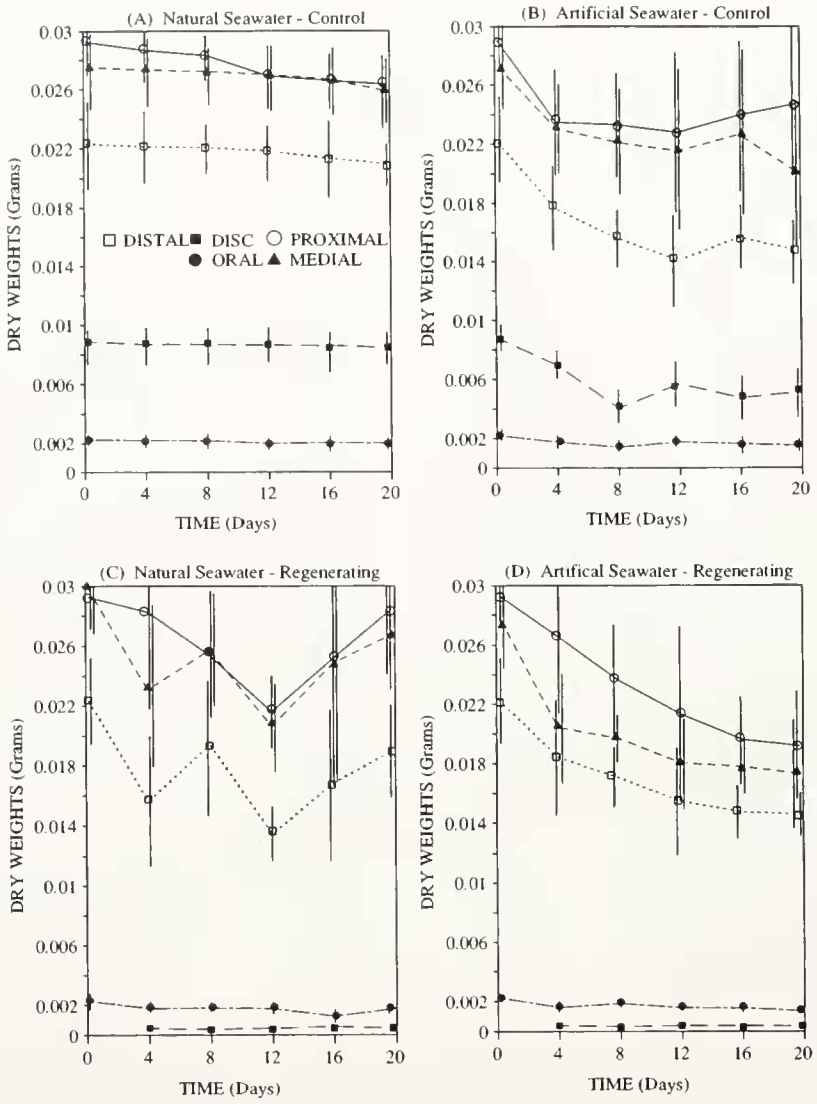

Figure 2. Changes in dry weights by body parts during early disc regeneration. (A) Natural seawater control group. (B) Artificial seawater control group. (C) Natural seawater regenerating group. (D) Artificial seawater regenerating group. Error bars represent 95% confidence intervals. Error bars and points offset slightly for graphical clarity.

tration over the course of the experiment ($P < 0.001$ in both). The protein concentration increased at the same rate in both the NR and AR groups until day eight, after which the NR group continued to gradually increase while the AR group began to decline. By the end of 20 days, the protein concentration in the AR group was the same as its initial (day zero) protein concentration.

The change in protein concentration over time by treatment group and body part is shown in Figure 6. There was no change in protein concentration in any body part in the NC group over the course of the experiment ($P > 0.05$) (Fig. 6A). The AC group lost protein in significant amounts from the disc ($P = 0.0012$), distal arm fractions ($P < 0.0001$), and oral frame ($P < 0.0001$). The protein concentration of the medial and proximal arm fractions did not change ($P = 0.0675$, $P = 0.7822$, respectively) (Fig. 6B). There was no change in the protein concentration of any arm fractions in the NR group ($P > 0.05$), but the oral frame lost significant amounts of protein relative to its dry weight ($P = 0.0003$), while the disc rapidly increased in protein concentration ($P < 0.0001$) (Fig. 6C). The AR treatment group lost protein from all non-regenerating body parts ($P < 0.05$) (Fig. 6D). The protein concentration of the disc in the AR group increased rapidly until day 12 ($P < 0.0001$), then fell off rapidly through day 20 ($P < 0.0001$). The rate of increase to day 12 was the same as in the NR treatment ($P > 0.05$). The rate of protein loss from the oral frame was slower in the AR

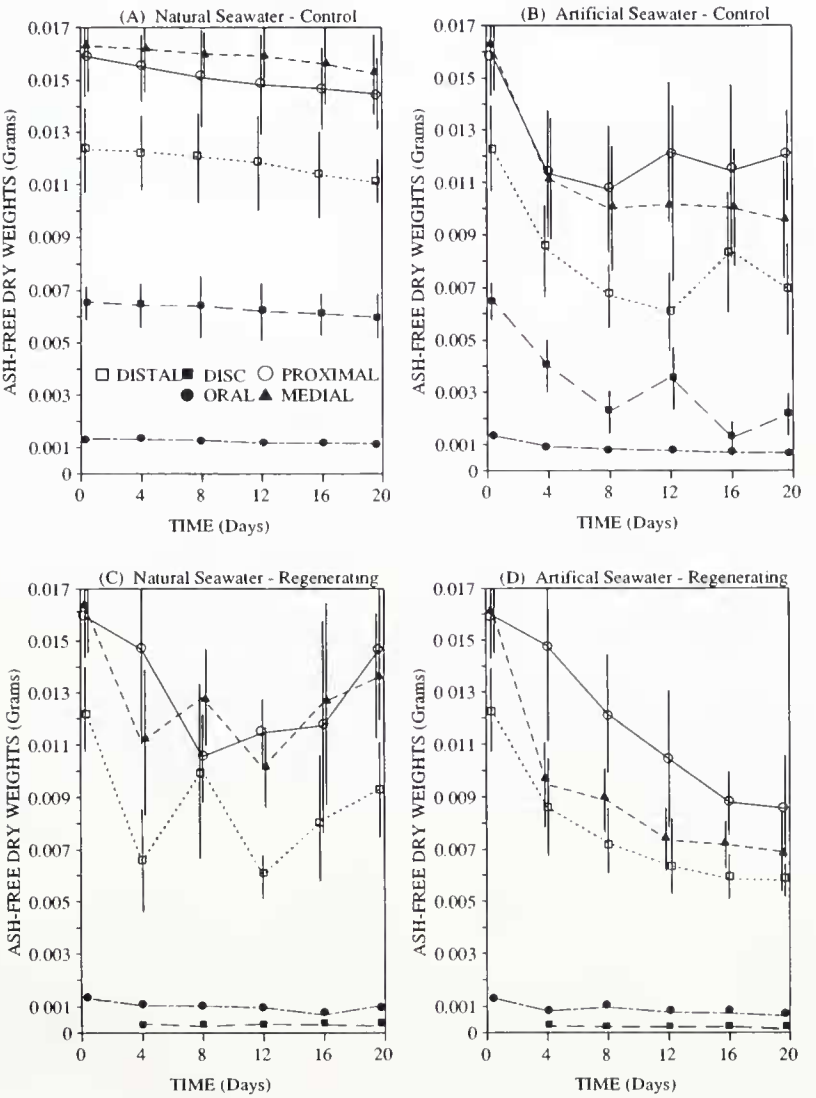

Figure 3. Changes in ash-free dry weights by body parts during early disc regeneration. (A) Natural seawater control group. (B) Artificial seawater control group. (C) Natural seawater regenerating group. (D) Artificial seawater regenerating group. Error bars represent 95% confidence intervals. Error bars and points offset slightly for graphical clarity.

and NR groups than in the AC group ($P < 0.0001$), but loss occurred throughout the experiment, whereas the AC group stopped losing protein from the oral frame at about day 8. The AR and NR treatment groups lost protein from the oral frame at the same rate throughout the experiment ($P = 0.0931$). The AR treatment lost protein from the distal arms at a higher rate than did the AC treatment group ($P < 0.0001$).

Carbohydrate content changes. The results of the total body carbohydrate assays are graphed by day in Figure 5B. The NC and NR groups did not exhibit any significant change in total carbohydrate concentration with time ($P = 0.0877$, $P = 0.4784$). The AC and AR groups did exhibit changes in total carbohydrate concentration ($P = 0.0063$, $P < 0.0001$) over the course of the experiment. There was no difference in the rate of loss between the AC and AR groups ($P = 0.5675$).

The changes in carbohydrate concentration of the various body parts with time in the different treatments is graphed in Figure 7. The NC, AC, and NR groups lost significant amounts of carbohydrates only from the oral frame ($P = 0.0173$, $P = 0.0002$, $P = 0.0075$, respectively). Although there were fluctuations in the carbohydrate concentration of the other non-regenerating body parts in each of these groups, they did not represent significant changes in concentration with time (Fig. 7A, B, C). The AR group lost significant amounts of carbohydrates from the distal and medial arm parts and the oral frame (P

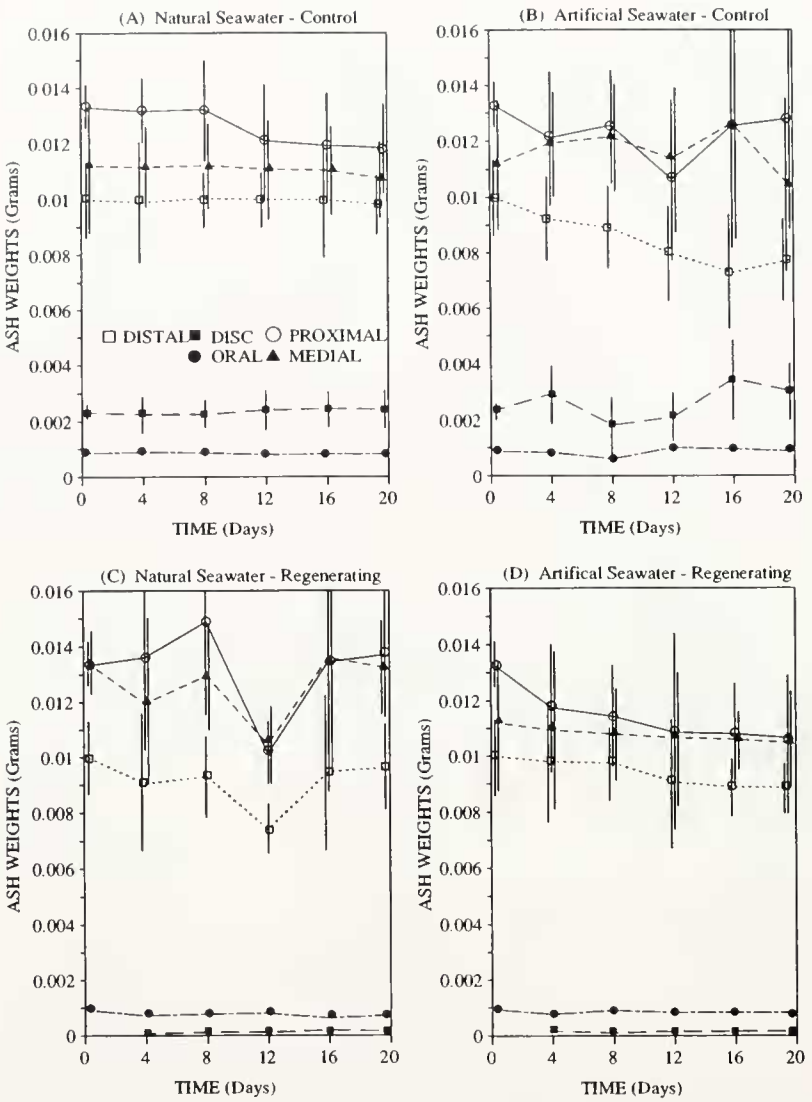

Figure 4. Changes in ash weights by body parts during early disc regeneration. (A) Natural seawater control group. (B) Artificial seawater control group. (C) Natural seawater regenerating group. (D) Artificial seawater regenerating group. Error bars represent 95% confidence intervals. Error bars and points offset slightly for graphical clarity.

< 0.05). The proximal arm parts did not exhibit a significant change, although there appeared to be a gradual decline in carbohydrate concentration ($P = 0.0623$, Fig. 7D). Both the NR and AR groups exhibited a rapid increase in the carbohydrate content of the regenerating disc, with rate of increase being the same in both groups through day 12. After day 12, the disc continued to increase in carbohydrate concentration in the NR group, while the carbohydrate concentration in the disc tissue of the AR group began to decline. The rate of decline in oral frame carbohydrate concentration was identical across all treatments ($P > 0.05$) until day 20, when the NC treatment was different from the AC, NR, and AR treatments, which were still the same ($P < 0.05$).

Lipid content changes. The changes in total body lipid concentration over time are shown in Figure 5C. There were no significant changes in the total lipid concentration within any of the treatments with time ($P > 0.05$). Between treatments, the NC and AC treatments were identical on all days. In addition, the NR and AR treatments were identical through day 12. The NR treatment was different from the AR treatment and the same as the NC and AC treatments at day 16. All treatments had the same lipid concentrations by day 20.

The changes in lipid concentration by day and body part are illustrated in Figure 8. There was no significant change in lipid concentration in any non-regenerating body part in the NC, AC, and NR groups ($P > 0.05$) (Fig.

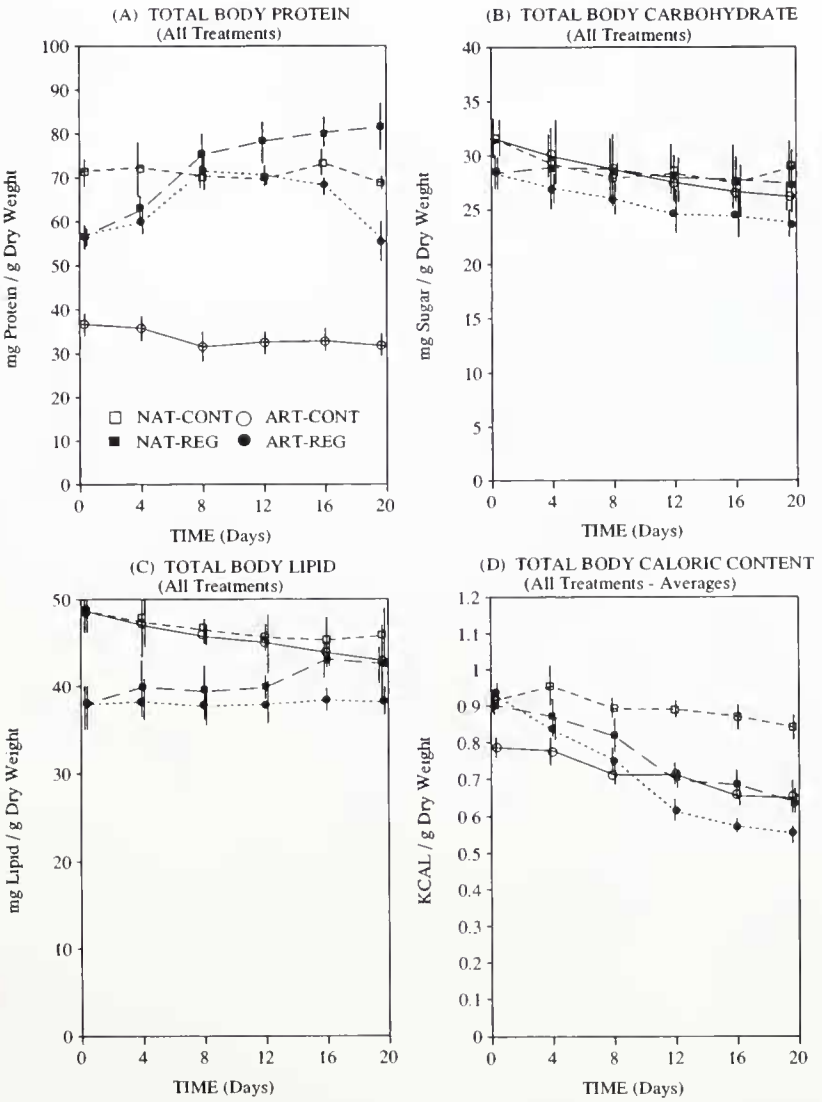

Figure 5. Total body biochemical concentration changes during early disc regeneration. (A) Total body protein by treatment group. (B) Total body carbohydrate by treatment group. (C) Total body lipid by treatment group. (D) Total body caloric content (calculated) by treatment group. Error bars represent 95% confidence intervals. Error bars and points offset slightly for graphical clarity.

8A, B, C). Although the AC and NR groups showed a constant decline in lipid concentration in all body parts except the regenerating disc of the NR treatment, the overall changes were not statistically significant. The AR group showed a significant decrease in lipid concentration in the medial arm fraction ($P = 0.0235$) as well as the same non-significant concentration decline in all other non-regenerating body parts shown by the AC and NR groups. The NR and AR groups exhibited rapid increases in lipid concentration in the disc tissue fragment, which were the same through day 16 ($P > 0.05$). The NR group had a higher lipid concentration in the disc fraction by day 20 ($P = 0.2430$).

Caloric content changes. Caloric values presented here were calculated from the biochemical data using caloric-conversion values (protein, 5.65 kcal/g; carbohydrate, 4.10 kcal/g; lipid, 9.45 kcal/g;) (Brody, 1964; Ekert and Randall, 1978). Although the current trend in physiological research is to use the SI unit of energy (joules), we determined energy content as calories and present the data here in calories for ease of comparison with previous literature. However, one calorie equals 4.184 joules (Crisp, 1984), so direct conversion between units is relatively simple. Although there is potential for error in using calculated values instead of real values for caloric content (Giese, 1966; Cummins and Wuy-

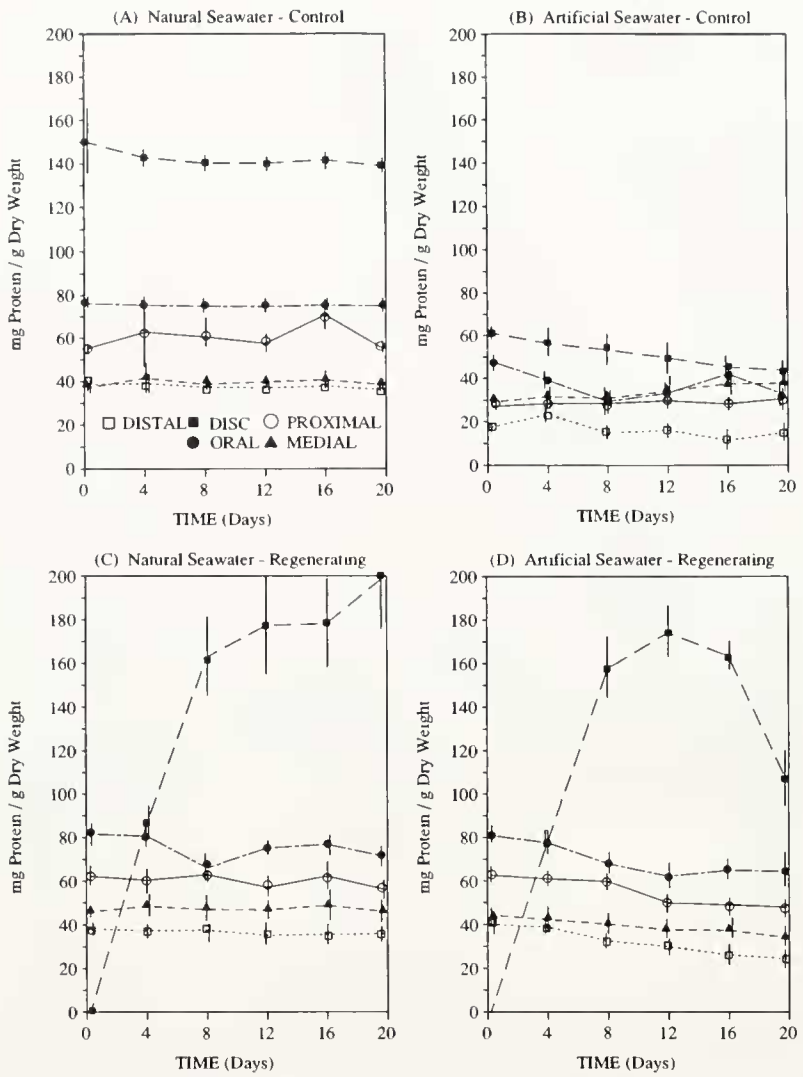

Figure 6. Protein content changes by body part during early disc regeneration. (A) Natural seawater control group. (B) Artificial seawater control group. (C) Natural seawater regenerating group. (D) Artificial seawater regenerating group. Error bars represent 95% confidence intervals. Error bars and points offset slightly for graphical clarity.

check, 1971; Feral, 1985), the calculated caloric values are probably closer to the "true" values than the actual calorimetry data due to procedural errors in obtaining the micro-bomb calorimetry data and the resulting wide variations in the actual caloric values. The total calculated caloric content of the body in the different treatments is illustrated in Figure 5D. With the exception of day four in the AC group (which was only different from day 20), there were no statistically significant differences in caloric content with time in either of the NC and AC groups ($P > 0.05$). The caloric content of the NR and AR groups declined constantly ($P < 0.0001$), with the AR group losing caloric content faster than the NR group ($P < 0.0001$).

The caloric content changes with time by body part are diagrammed in Figure 9. The natural seawater control group lost calories only in the disc fraction ($P = 0.0310$) (Fig. 9A). All other body parts maintained their caloric levels ($P > 0.05$). The artificial seawater control group lost calories only from the disc and oral frame ($P = 0.0045$, $P = 0.0050$), not the arm fractions ($P > 0.05$) (Fig. 9B). The NR treatment group lost calories from the oral frame and distal arm fractions ($P = 0.0017$, $P = 0.0064$) but not the medial and proximal arm fractions ($P > 0.05$) (Fig. 9C). The AR group lost calories from every non-regenerating body part ($P < 0.0001$) (Fig. 9D). The NR and AR groups both increased the caloric content of their disc tissue until day 16. By day 20, the AR group had begun

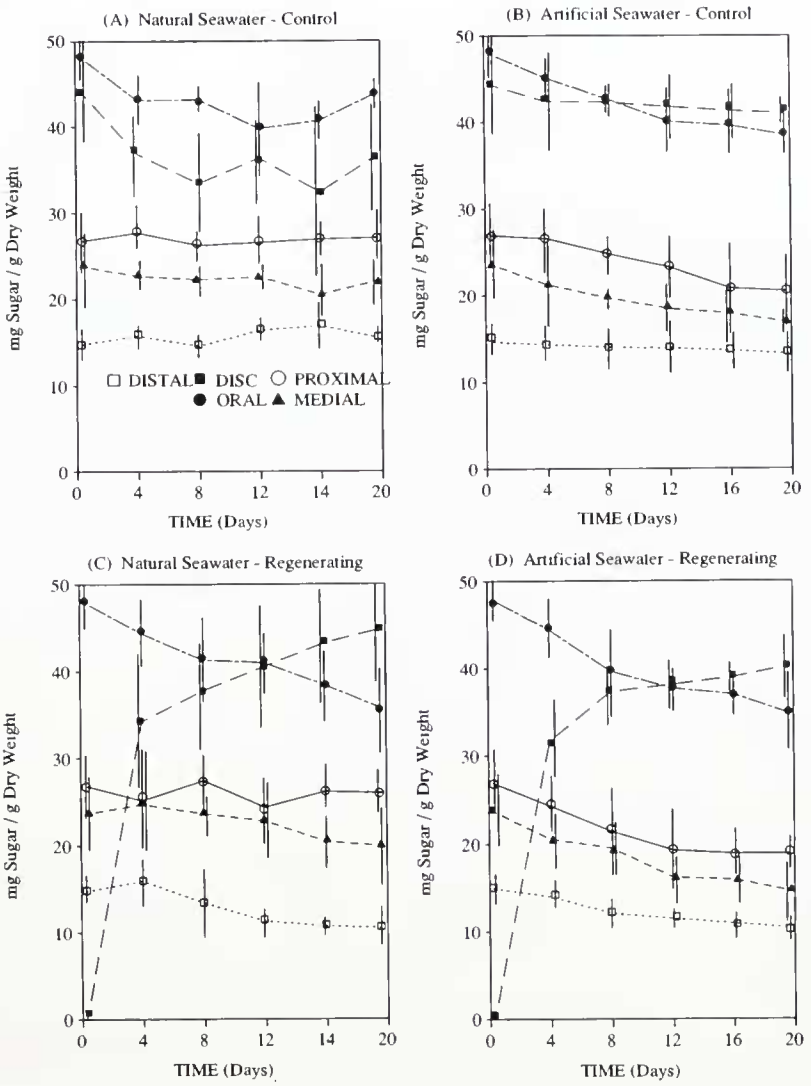

Figure 7. Carbohydrate content changes by body part during early disc regeneration. (A) Natural seawater control group. (B) Artificial seawater control group. (C) Natural seawater regenerating group. (D) Artificial seawater regenerating group. Error bars represent 95% confidence intervals. Error bars and points offset slightly for graphical clarity.

to lose calories from the disc tissue, whereas the NR group continued to add calories to the disc tissue. The rate of increase in caloric content was the same in the NR and AR groups through day 12.

Rate of nutrient translocation

All brittlestars took up statistically significant amounts of ^{14}C-leucine and ^{14}C-glucose during the pulse portion of the experiment (Fig. 10, 11). Counts of the individual body parts indicated that all body parts absorbed label in approximately the same quantities per gram of dry body weight ($P = 0.5740$). However, the animals only accumulated significant amounts of ^{14}C-palmitic acid in the disc region of the body. This result was somewhat unexpected, because other echinoderms are known to take up lipids, especially exogenous palmitic acid, from their environment across their dermal surfaces (Beijnink and Voogt, 1984).

During the post-absorption portion of the experiment, ^{14}C-leucine and ^{14}C-glucose label counts decreased rapidly and in approximately linear fashion in all the experimental treatments (Fig. 10, 11). ^{14}C-palmitic acid concentration changes were not followed because the animals failed to take up the material in non-regenerating body parts. Counts of the individual body parts indicated that ^{14}C-leucine and ^{14}C-glucose labels were lost in approximately the same proportions from all non-regenerating fractions

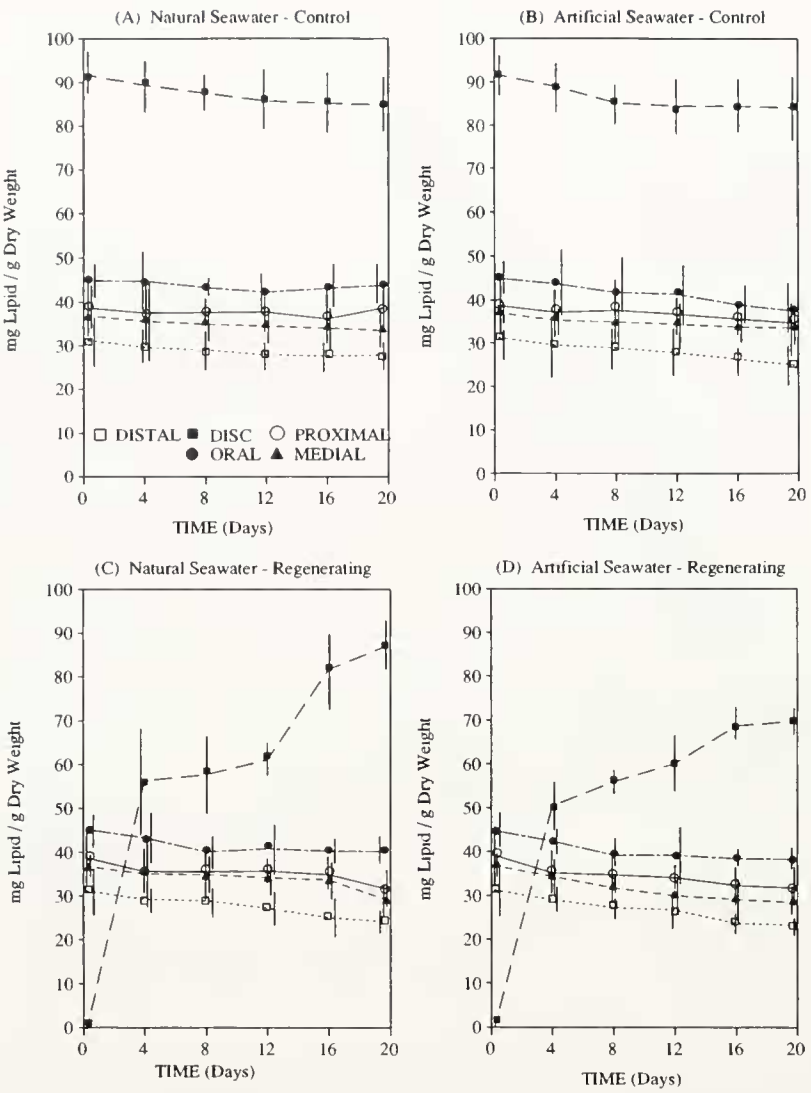

Figure 8. Lipid content changes by body part during early disc regeneration. (A) Natural seawater control group. (B) Artificial seawater control group. (C) Natural seawater regenerating group. (D) Artificial seawater regenerating group. Error bars represent 95% confidence intervals. Error bars and points offset slightly for graphical clarity.

of the body, including the disc of intact specimens. However, little of the label lost from the non-regenerating tissues of regenerating animals was incorporated into the regenerating disc tissue. Although counts of the regenerating disc tissue showed that some radiolabel was incorporated into the disc tissue, the levels were not significantly different from background counts throughout the course of the experiment ($P > 0.05$).

Discussion

The experimental treatments used to study the amount of nutrients translocated during disc regeneration can be described in terms of nutrient availability. The NC group represented control animals that were given access to dissolved organic material (DOM), but not particulate food, to determine the effect of maintenance metabolism on the body's biochemical composition when both stored nutrient catabolism and DOM uptake were available as energy sources. The AC group represented control animals that had to rely on stored nutrients alone to supply energy for maintenance. The NR group were animals that had to supply energy for both maintenance metabolism and regeneration, as well as building materials for regeneration. These animals had access to both stored nutrients and DOM uptake sources of nutrients. The AR group represented animals that had to both maintain metabolism and regenerate

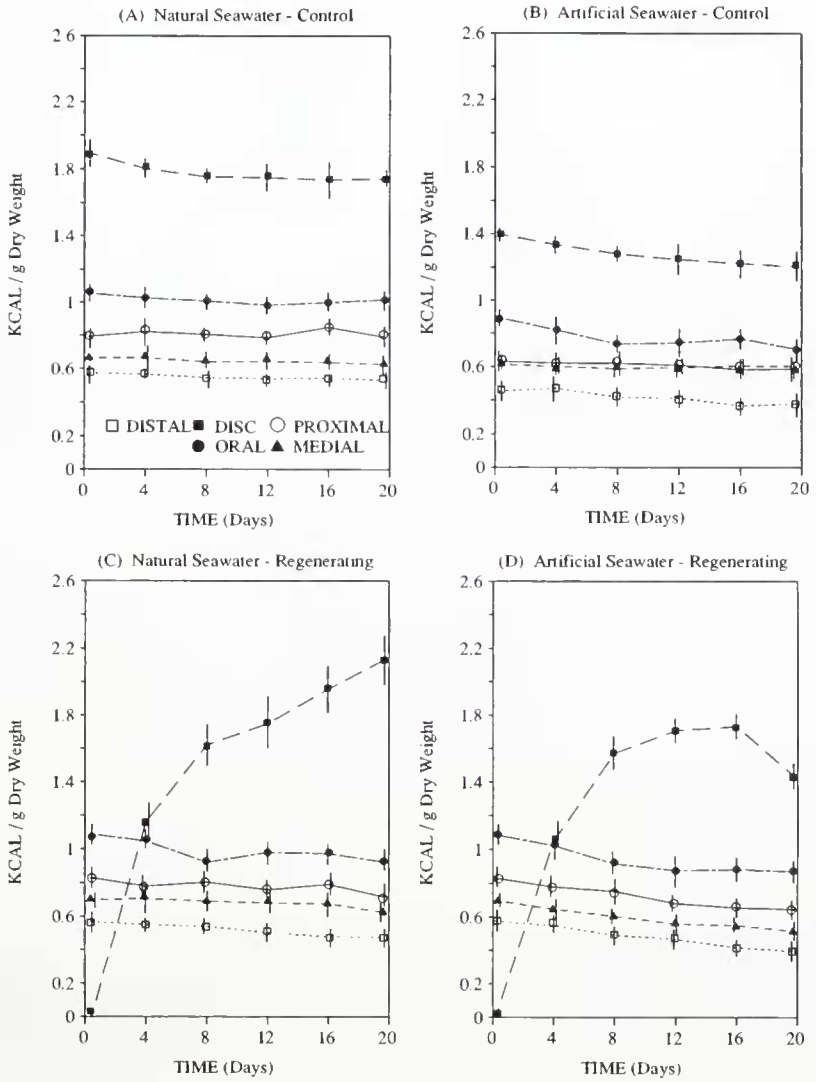

Figure 9. Tissue caloric content changes by body part during early disc regeneration. (A) Natural seawater control group. (B) Artificial seawater control group. (C) Natural seawater regenerating group. (D) Artificial seawater regenerating group. Error bars represent 95% confidence intervals. Error bars and points offset slightly for graphical clarity.

in the absence of any external nutrient source (*i.e.*, only stored nutrients were available).

Under natural seawater control conditions, animals survived for at least four weeks (one week of acclimation plus three weeks of experimentation) with no significant change in the overall biochemical composition of the body. The only localized changes in body constituents occurred in the oral frame region, which lost small amounts of carbohydrates during the experiment. Although the animals probably lost stored nutrients from all body parts under these conditions, the losses were below the limits of detection. The total energy content of the animal did not change. The only localized caloric content change occurred in the disc region and could not be attributed to changes in any measured biochemical component. This indicates that, although animals deprived of particulate food may be stressed, they probably are not starving (*i.e.*, they are obtaining nutrients by direct uptake from the environment). This result is consistent with previous studies showing that echinoderms, including brittlestars, can obtain up to 58% of their energetic requirements from DOM (Ferguson, 1982a, b; Feral, 1985; Lawrence, 1987; Clements, 1988).

When deprived of all exogenous food (artificial seawater treatments), control animals initially lost stored material at a rapid rate. The material was lost from the disc, oral frame, and distal arm regions of the body, and was attributable to losses of protein and carbohydrates, but not

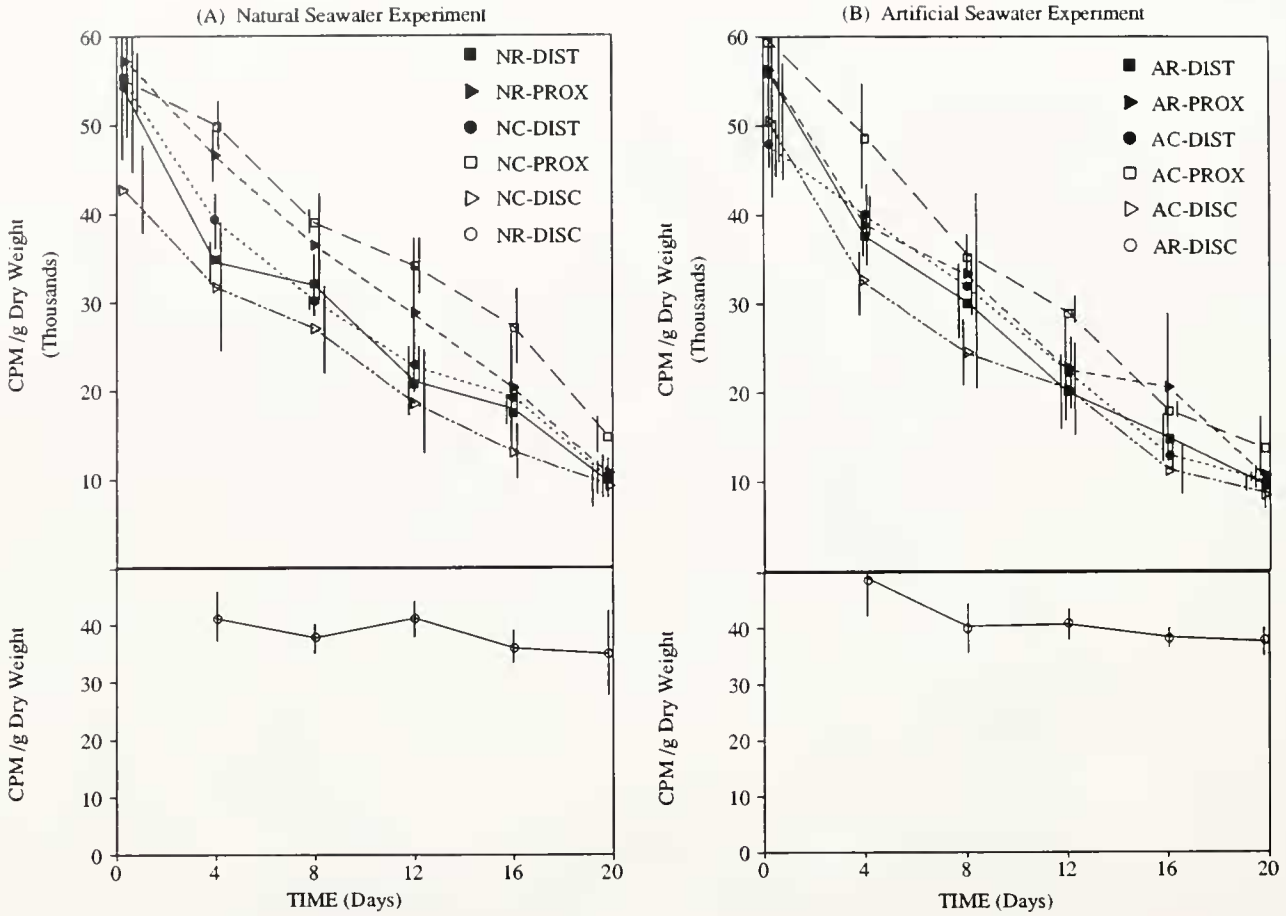

Figure 10. ^{14}C-Leucine tracer content of tissues during early disc regeneration. (A) Natural seawater experiment. (B) Artificial seawater experiment. AC = Artificial seawater control, AR = Artificial seawater regenerating, NC = Natural seawater control, NR = Natural seawater regenerating, DIST = Distal arm, PROX = proximal arm and oral frame. Error bars represent 95% confidence intervals. Error bars and points offset slightly for graphical clarity.

lipids. The loss of total caloric content in the various body parts followed the same pattern, with no significant loss in any other body parts. After four days, these animals appeared to acclimate to the lack of food such that the rate of overall materials loss was reduced; (*i.e.*, they apparently adjusted to food deprivation by reducing their consumption of stored material). The temporal pattern of material loss may also represent a rapid initial use of stored resources followed by a breakdown of essential body tissues to maintain metabolism. As tissue mass decreased, the metabolic load due to those tissues decreased, and the rate of tissue loss declined. Because mass-specific metabolic rates were not obtained during this experiment, these observations could not be empirically verified.

There are two possible explanations for the spatial pattern of material loss in the artificial seawater control group. The first is that the disc, oral frame, and arm tips are preferentially resorbed when the animal is forced to catabolize tissue for maintenance. Turner and Murdoch (1976) described such a pattern of arm tissue loss during regeneration of the disc in *Ophiophragmus filograneus*. This mechanism would leave the majority of the arm tissue undisturbed so that normal feeding activity would not be impaired when feeding conditions improved. The second possibility is that the absolute rate of loss is the same from all body parts, but there is less material in the disc, oral frame, and arm tips to begin with, so the material available within them is exhausted sooner than that in other body parts. The latter possibility is the more likely, because the medial and proximal arms have the highest total amounts of all biochemical constituents (Table I).

Animals regenerating in natural seawater showed an initial decrease in organic mass followed by a gradual increase. This indicates that the use of material during early regeneration exceeded the rate at which DOM uptake from the medium could compensate for it, and thus must have been at least partially independent of external nutrient availability. Loss of organic material occurred in

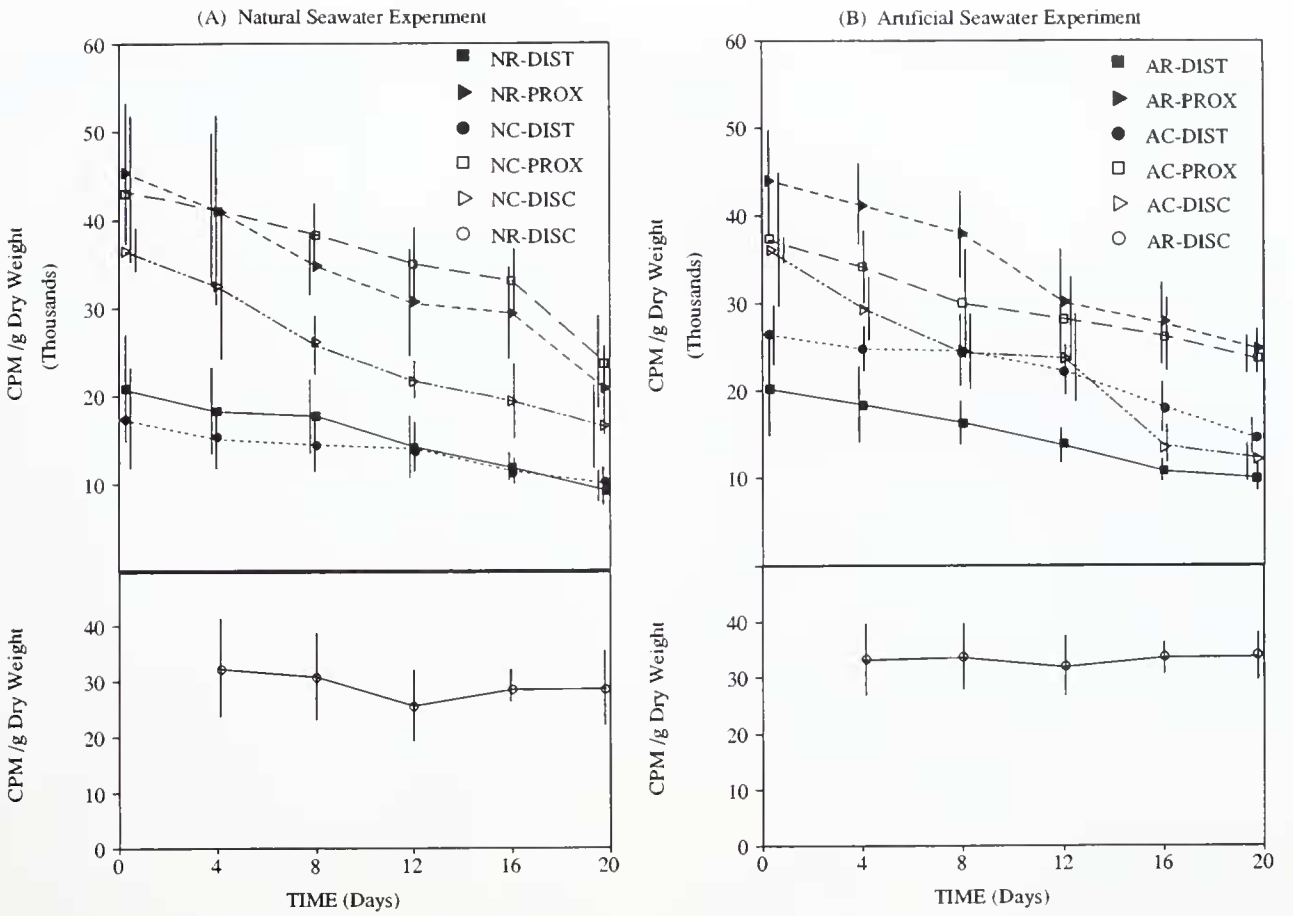

Figure 11. ^{14}C-Glucose tracer content of tissues during early disc regeneration. (A) Natural seawater experiment. (B) Artificial seawater experiment. AC = Artificial seawater control, AR = Artificial seawater regenerating, NC = Natural seawater control, NR = Natural seawater regenerating, DIST = Distal arm, PROX = proximal arm and oral frame. Error bars represent 95% confidence intervals. Error bars and points offset slightly for graphical clarity.

all non-regenerating body parts, but the overall trend was similar to that exhibited by the artificial seawater control group in that most of the loss was from the oral frame and arm tips. The subsequent increase in organic material appeared to be localized in the arms. The relative protein content of the body increased constantly during regeneration, indicating either a net gain of protein during regeneration, or a loss of minerals as tissue breakdown occurred. This gain could be due to a net uptake of proteins (or amino acids) from the medium, or a combination of uptake and overall loss of other body biochemical constituents during regeneration. Although the carbohydrate and lipid content of the body did not change significantly over the same period, the latter explanation is more likely, because the animals lost total caloric content constantly during regeneration. The increase in organic material in the non-regenerating portions of the body after day 12 is problematical, because no corresponding increase in biochemical constituents in those parts could be demonstrated. This increase might be explained as the summation of non-significant increases in each biochemical constituent to make a significant increase in total organics.

Animals regenerating in the absence of exogenous nutrients constantly lost organic material from non-regenerating body parts. The rate of loss was relatively rapid through day 8, and slower from day 12 through day 20. This change was related to the constant decrease in protein, carbohydrate, and lipid content of the non-regenerating tissues. Although lipid content loss was statistically significant only in the medical portions of the arms, all non-regenerating body parts showed a trend toward lipid loss. The regenerating disc tissue increased in protein, carbohydrate, and lipid content through day 12, after which protein and carbohydrate content dropped dramatically, while lipid content remained the same or slightly increased (the continued proportional lipid increase was probably due to the loss of protein and carbohydrates). The caloric content of these animals dropped

constantly, and consistently faster than that in the experimental group regenerating in natural seawater. All non-regenerating parts of the body lost calories throughout the experiment. The caloric content of the disc increased through day 16, then dropped dramatically.

The data on the consumption of biochemicals and disc tissue production in the regeneration experimental groups, especially the artificial seawater regeneration group, seems to indicate that the process of regeneration runs at a set rate, and may be independent of the nutritional state of the animal (at least for the first two weeks of disc regeneration). A similar phenomenon has recently been reported in crinoids under field conditions (Meyer, 1988). These observations imply that early replacement of initial disc tissues and structures has priority over the maintenance of body mass. Since these observations coincide temporally with appearance of the functional gut (Dobson and Stancyk, in prep), one can conclude that the animal tries to replace the gut so it can feed again regardless of its initial nutritional state. Only when resources drop below some critical level (*i.e.*, the actual onset of starvation) do they stop regenerating the disc. This experiment should be repeated with animals that have been held without food sources for varying lengths of time to determine whether regeneration is even initiated after the critical point in the food withdrawal period has passed.

Regeneration appears to require a set amount of nutrients, which are transported from the deep tissues of all the non-regenerating body parts. If food is present (as DOM in this case) the loss of material due to translocation may be offset by uptake. Further, after the gut lining is reformed and becomes functional, ingestion of particulates, including small bacteria, may ameliorate the loss of stored nutrients.

A previous attempt to verify and quantify nutrient translocation into the disc from somatic body parts during disc regeneration in *M. gracillima* was unsuccessful (Clements, 1988). That study relied on the assumption that loss of organic material from the arms would result in a decrease in total arm size. This assumption was based on the results of Turner and Murdoch (1976) and the observation that echinoid test diameter decreases during starvation (Ebert, 1967). However, a loss of arm tissue without a reduction in overall arm size has been demonstrated in starving asteroids (Lawrence *et al.*, 1986). Thus, the internal soft tissues of asteroid arms are scavenged while leaving the calcified structures in place. Indeed, the arms of asteroids have been implicated as general nutrient storage organs (Beijnink and Voogt, 1984; Lawrence, 1987). If the non-regenerating body parts of *M. gracillima* are fulfilling a similar role, then translocation of organic material from the non-regenerating body parts should occur without an overall decrease in body part size or inorganic (=ASH) weight. The calcification of tissues in marine invertebrates is also a relatively expensive process compared to the production of soft tissues, due to the energetics of mineralization and the cost of producing the skeletal matrix (Simkiss, 1976; Palmer, 1983; Lawrence, 1987). Consequently, we would expect the calcified structures of *M. gracillima* to be conserved even as its soft tissues are degraded to supply catabolic and regenerative nutrients. Because the entire external surface of *M. gracillima* is covered with plate ossicles and spines, the shape and size of body parts would not change much as the soft tissues are degraded inside the structures. The absence of change in the ash weight of all the body parts of all animals in the current study supports this hypothesis.

Abnormal regeneration and death of specimens in the nutrient-enriched experimental groups is perplexing, but has been verified by repeated experimentation (Clements, 1988; K. Fielman, pers. comm.). Because preliminary experiments indicated that these conditions promoted bacterial growth (Clements, 1988), we took care to inhibit such growth by completely changing the medium each day. Several researchers have proposed that echinoderm regeneration requires the presence of functional nerve fibers that produce recognition and regulatory molecules (Bisgrove *et al.*, 1988; P. Mladenov, pers. comm.). Abnormally high ambient concentrations of nutrients (especially amino acids, which can act as neurotransmitters) may have directly affected the regeneration process by interfering with the actions of these recognition molecules.

Uptake of ^{14}C-leucine and ^{14}C-glucose indicated that dissolved organic material is taken up in statistically significant amounts in all treatments. The results agree closely with those of Clements (1988) for net uptake of the amino acids leucine and glycine by *M. gracillima*. However, her study showed significant retention of the labeled compound over time. The current results indicate that the initially retained labeled molecules are rapidly turned over or leaked back into the medium, with little permanent incorporation of the labeled molecules into the tissues and no translocation of the labeled material to the active regeneration site. The labeled compounds may have been transported in quantities below the detected threshold of the assay method. We do not know whether the loss of label from non-regenerating tissues is due to leakage or respiration, because the experiment was not designed to test for respired $^{14}CO_2$ or for an increase in the label content of the medium with time. We would understand this process better if the ^{14}C-leucine, ^{14}C-glucose, and $^{14}CO_2$ evolved in the medium during the post-absorption portion of the experiment had been assayed to determine what fraction of the material taken up by the animals was catabolized or leaked out.

The absence of detectable translocation of radiolabeled material into regenerating tissue indicates that, if the labeled material is not simply leaking out of the body [which

is not expected to be the case based on the results obtained by Clements (1988)], then the material may have been absorbed only into the surface tissue layers of the body, and not subsequently transported into the deeper tissues. Several investigators have proposed such DOM absorption as a mechanism by which echinoderms, which have poor circulatory systems, maintain their external tissues (Ferguson, 1982b; Bamford, 1982). In these models, DOM feeds the external tissues, but is not transported into the deep tissues, whereas material ingested and digested is not transported to the surface layers but supplies nutrients only to the internal tissues. Because the current results, and the results of previous work on regeneration (Dobson and Stancyk, in prep), indicate that nutrients are translocated from the deep tissues of the non-regenerating body parts—probably by coelomocytes of the water-vascular system—the lack of label in the regenerating disc may be ascribed to its inability to migrate into the deep tissues and thus to be available for regeneration.

Disc autotomy is probably a predator avoidance mechanism (Turner *et al.*, 1981). Because the disc (or at least the gut) is needed for feeding, some mechanism should be available to replace it after escape-response disc autotomy, irrespective of the nutritional state of the animal. Such an effect has been demonstrated in this and a previous set of experiments (Dobson, Stancyk, and Clements, in prep). In addition, because *M. gracillima* is a seasonal spawner (pers. obs.), selection for rapid replacement of the disc structures to facilitate replacement of gonads and gametes would be expected. Because these animals lose up to one-fourth of their available body organic mass during early regeneration, a massive amount of body reserves must enter the process. However, a significant amount of the reserves must be used for maintenance metabolism. This study shows that, although these animals do have some energy storage resources (because the starving and regenerating animals still produce disc tissue), there is still no specific nutrient storage organ or tissue. Without additional exogenous nutrient input, these stores are depleted within about two weeks, a sufficient time for replacement of the gut and initiation of feeding, even when particulate and dissolved exogenous organic material is absent.

Acknowledgments

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